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Discussion on research methods of bacterial resistant mutation mechanisms under selective culture—uncertainty analysis of data from the Luria-Delbrück fluctuation experiment

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Whether bacterial drug-resistance is drug-induced or results from rapid propagation of random spontaneous mutations in the flora prior to exposure, remains a long-term key issue concerned and debated in both genetics and medicinal fields. In a pioneering study, Luria and Delbrück exposed *E. coli* to T1 phage, to investigate whether the number of resistant colonies followed the Poisson distribution. They deduced that the development of resistant colonies is independent of phage presence. Similar results have since been obtained on solid medium containing antibacterial agents. Luria and Delbrück's conclusions were long considered a gold standard for analyzing drug resistance mutations. More recently, the concept of adaptive mutation has triggered controversy over this approach. Microbiological observation shows that, following exposure to drugs of various concentrations, drug-resistant cells emerge and multiply depending on the time course, and show a process function, inconsistent with the definition of Poisson distribution (which assumes not only that resistance is independent of drug quantity but follows no specific time course). At the same time, since cells tend to aggregate after division rather than separating, colonies growing on drug plates arise from the multiplication of resistant bacteria cells of various initial population sizes. Thus, statistical analysis based on equivalence of initial populations will yield erroneous results. In this paper, 310 data from the Luria-Delbrück fluctuation experiment were reanalyzed from this perspective. In most cases, a high-end abnormal value, resulting from the non-synchronous variation of the two above-mentioned time variables, was observed. Therefore, the mean value cannot be regarded as an unbiased expectation estimate. The ratio between mean value and variance was similarly incomparable, because two different sampling methods were used. In fact, the Luria-Delbrück data appear to follow an aggregated, rather than Poisson distribution. In summary, the statistical analysis of Luria and Delbrück is insufficient to describe rules of resistant mutant development and multiplication. Correction of this historical misunderstanding will enable new insight into bacterial resistance mechanisms.

bacteria, mutation, random process, Poisson distribution, statistical test, aggregated distribution

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Low-frequency spontaneous mutations arise from changes in DNA sequence such as substitutions, insertions and deletions, which occur in the absence of exogenous DNA damage factors (e.g., mutagens). Induced mutations occur under selection pressure or exposure to a threat. Studies of mutant cells are typically based on mutant phenotype observed under certain conditions. Similarly, bacterial mutants are often investigated by growing colonies on agar plates under selective or non-selective conditions. In general, two types of mutants are not readily distinguishable by their colony phenotype, so it is difficult to determining whether resistance arises from spontaneous or induced mutation just

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by the mutant phenotype.

In genetics, DNA sequence changes are regarded as necessary but not sufficient conditions for phenotypic expression. In epigenetics, on the other hand, expression of partial phenotypes is thought to not depend on changes of DNA sequences [1]. The inference of DNA sequence changes via phenotypic development is extremely complex, and involves mutation mechanisms (for example, prolonged antibacterial drugs usage as a progenitor for the emergence and propagation of bacterial mutant strains) under selective conditions, which has always been controversial. In 1943, Luria and Delbrück calculated the mean and variance of *Escherichia coli* colonies grown in the presence of T1 bacteriophage, and inferred that the development of T1 phage-resistant mutants was independent of phage presence [2]. Luria and Delbrück's conclusion was later supported by the spread plate experiment [3] of Newcombe and the re-plate experiment [4] of Lederberg, both of which reported *E. coli* resistance to T1 phage and streptomycin. Subsequent experiments revealed that bacteria develop resistance to a variety of phages (T1–T7), and to drugs such as penicillin, streptomycin and sulfonamides, regardless of phage and drug presence. Such harmful agents kill sensitive cells only, so that pre-existing resistant mutants can multiply unimpeded to detectable levels [5,6]. This conjecture has become the popular mainstream view in microbiology and genetics.

However, in 1988, Cairns and colleagues observed that when they spread *E. coli* lacZ^- mutants onto nonlethal selective plates (with lactose as sole carbon source), the number of lacZ^+ back mutations that could form colonies had increased markedly two days later. During the first 48 hours, the number of colonies growing on the plates did not follow Poisson distribution. After 48 hours, during the long-term culture, more and more lacZ^+ colonies appeared on the plates, the number of lacZ^+ colonies followed Poisson distribution. Combined with other genetic evidences, Cairns *et al*. [6] proposed that the presence of lactose accelerated the reversion frequency of lacZ^- strains. They suggested that "under nonlethal selective conditions, bacteria are capable of choosing mutation benefiting survival". Thus, when exposed to nonlethal selective pressure for a sufficiently long time, bacteria can mutate at high frequency, far exceeding that of spontaneous mutation, to maximize their survival chances. This phenomenon, known as adaptive mutation, has been widely proven and has initiated the debate between Neo-Lamarckism and Neo-Darwinism [5–7]. The resistant mutants followed or deviated from the Poisson distribution, has been regarded as the mathematical basis of the criterion for differentiating adaptive mutation under the selective conditions and spontaneous mutation under non-selective conditions.

In Luria and Delbrück's experiments, *E. coli* B cultured for different periods were spread-plated onto LB agar and sprayed with sufficiently lethal doses of T1 phage. Following culture, resistant colonies were found on every plate. During pre-incubation, *E. coli* B sensitive cells multiplied exponentially and regularly. Fluctuations in resistant cell numbers are irrelevant to cells in this state, although spontaneous mutation could happen randomly at any time prior to phage exposure. The mutants could then multiply to yield significant numbers of advantageous mutant daughter cells, a process known as jackpot [2]. Since the inoculation fluid was sampled randomly from series of independent pre-culture solutions containing jackpot mutant cells, the fluctuation of mutant colonies on the plates was amplified. These fluctuations were later termed the Luria-Delbrück distribution. Whether mutant strains obey the Luria-Delbrück distribution in independent culture has become the accepted criterion by which to distinguish spontaneous from adaptive mutation.

The Luria-Delbrück hypothesis states that, if mutation is a response to phage presence, every plate inoculated from a given culture should grow a similar number of mutant colonies, since the bacterial cells will multiply similarly and will experience similar invasiveness of phages. The numbers of colonies on the plates should then approach the Poisson distribution with a variance to mean ratio of approximately one.

Spontaneous and induced mutations are both small-probability events that are difficult to delineate by phenotype. Distinguishing between the two mutations is aided by statistical analysis of colony number fluctuations under different culture conditions. Thus, the Luria-Delbrück fluctuation experiment has been hailed as a model example of how statistical methods can solve biological problems. However, in that experiment, mutant cells in independent liquid cultures (small test tubes) arise in two ways: via new mutation events, or via division and multiplication of pre-existing mutants in the cultures. For this reason, mutant cell number in cultures sampled and spread onto plates is influenced by the number and composition of cells in the cultures and by culturing period. The former is extremely difficult to quantify accurately. The lack of agreement on Luria-Delbrück distribution provokes further controversy among biologists and biological statisticians, Luria-Delbrück distribution has been analyzed by conditional moment estimation, minimum χ^2 estimation and maximum likelihood estimation, and others [8–21], a FALCOR net based on the Luria-Delbrück fluctuation test has been established [22]. Because the fluctuation test is not only necessary for accurately determining mutation rate, but also involves the relationship between antibacterial agent utilization and bacterial resistance mutation, the Luria-Delbrück distribution problem remains unsolved. Elucidation of this problem would improve the reliability of statistical analysis in solving other biological problems.

Combining related progress [23–33] in microbiology and molecular biology in recent years and the studies conducted in our laboratory [34–43], this paper first analyzes microbi-

ological evidences for the Luria-Delbrück hypothesis, and discusses limitations and errors in applying the hypothesis to observed data and statistical analysis. It proceeds with a statistical re-analysis of the Luria-Delbrück data, from which new inferences are made. It also presents corresponding microbiological analysis and new interpretation of the Newcombe Spread Plate test, and suggests strategies for distinguishing between spontaneous and induced mutations.

1 Limitations of Luria-Delbrück hypothesis

1.1 Luria-Delbrück experimental design

Luria and Delbrück designed a complex multi-factor conditional test to prove their hypothesis [2]. The test consisted of four groups denoted A, B, C and D, and 13 samples, yielding a total of 310 sampled data [2]. To ensure that no resistant cells were initially present in independent cultures, the initial cell number in all tests was restricted to 50–100 CFU/culture (where CFU denotes colony forming units). At the end of culture, when cell numbers had expanded to 1×10^8–50×10^8 CFU mL^{-1}, a specified volume of liquid (e.g., 0.05 or 0.08 mL) from each culture was spread onto LB plates. After spraying with effective doses of T1 phage, the plates were incubated for 24 or 36 h at 37°C, and resistant colonies on each plate were counted. The Luria-Delbrück experiment included two kinds of sampling modes (Figure 1). In group A, sampling was repeated 10 times with sampling volume of 0.5 mL from one culture (10 mL per culture), sampling 10 times per culture. In group B, sampling was repeated 5–10 times with sampling volume of 0.05 mL from the 5–10 cultures (10 mL per culture), sampling one time per culture, similarly in group C and D (Table 1).

Repeated sampling and non-repeated sampling are two common ways of sampling finite populations; samples obtained through repeated sampling are random and independent, while the reverse is true for non-repeated sampling. Samples from both techniques follow a specific probability distribution. Different sampling modes require different methods for computing expectation value and variance in sample mean.

Treatment effects can be correctly inferred only when the number of regional test repetitions is sufficiently large that the degree of freedom of the error equals or exceeds 10. If

Table 1 Summary of the data obtained in the Luria-Delbrück fluctuation experiment [2][a)]

Group A

Sample No.	A-1	A-2	A-3
Volume of cultures (mL)	10	10	10
Volume of samplings (mL)	0.5	0.5	0.5
Number of samplings	10	10	10
Sampling mode		Sampling mode A (Figure 1A)	
Resistant colonies per sample x_i	13	44	1
	13	46	2
	14	47	2
	14	48	2
	15	49	2
	15	51	4
	16	52	4
	20	56	4
	21	56	5
	26	65	7

Group B

Sample No.	B-1	B-2	B-3	B-4	B-5
Volume of cultures (mL)	10	10	10	10	10
Volume of samplings (mL)	0.05	0.05	0.05	0.05	0.05
Number of samplings	9	8	10	10	5
Sampling mode		Sampling mode B (Figure 1B)			
Resistant colonies per sample x_i	3	7	10	5	13
	10	17	12	6	28
	10	17	23	6	35
	14	20	30	8	38
	17	29	40	10	107
	17	30	45	10	
	18	31	51	13	
	27	41	57	15	
	125		173	24	
			183	165	

Group C

Sample No.	C-1	C-2	C-3
Volume of cultures (mL)	0.2	0.2	0.2
Volume of samplings (mL)	0.08	0.08	0.05
Number of samplings	20	12	19
Sampling mode	Sampling mode B (Figure 1B)		
Resistant colonies per sample x_i	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	1	0
	0	1	0
	0	3	0
	0	4	0
	0	7	0
	0	48	0
	1	303	0
	1		1
	3		1
	5		8
	5		11
	6		15
	35		17
	64		19
	107		

Group D

Sample No.	D-1	D-2
Volume of cultures (mL)	0.2	0.2
Volume of samplings (mL)	0.05	0.2
Number of samplings	100	87
Sampling mode	Sampling mode B (Figure 1B)	
Resistant colonies per sample x_i	0(57)	0(29)
	1(20)	1(17)
	2(5)	2(4)
	3(2)	3(3)
	4(3)	4(3)
	5(1)	5(2)
	6–10(7)	6–10(5)
	11–20(2)	11–20(6)
	21–50(2)	21–50(7)
	51–100(0)	51–100(5)
	101–200(0)	101–200(2)
	201–500(0)	201–500(4)
	501–1000(1)	501–1000(0)

a) Data are divided into groups based on differences in sample sizes, sampling frequency and sampling modes. A schematic of the sampling modes is shown in Figure 1.

this is not the case, the F value does not meet the required level of significance to verify a cause-and-effect. The reason is that the smaller the degree of freedom of error, the larger the variance in the error. Since the F value is precisely the ratio of treatment variance to error variance, a large variance in the error may decrease the F value to below its significant level, by which the essential differences of treatments are distinguished. We note that the sampling case n of both Group A and Group B in the Luria-Delbrück experiment is less than 10.

1.2 Hypothesize that T1 phage is sufficiently lethal to *E. coli* B to exclude the possibility of induced mutations

Luria and Delbrück interpreted their experimental results under the hypothesis that phage T1 is a lethal factor to *E. coli* B. In the presence of sufficient T1, if no resistant cells exist in the inoculation flora, all sensitive cells will become infected and lysed; if resistant cells are present, they can survive and multiply to become visible colonies during plate culture. Therefore, according to the hypothesis, all colonies

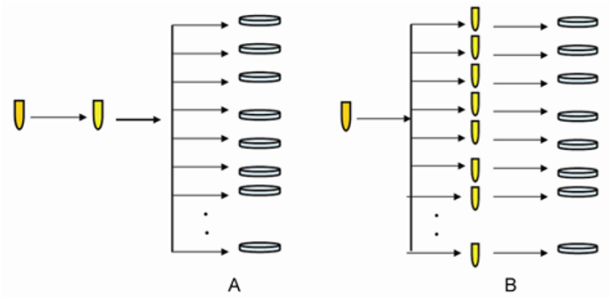

Figure 1 Sampling mode scheme in the Luria-Delbrück fluctuation experiment. T1-sensitive *E. coli* culture was stored in orange tubes. Following cultivation, >1000 CFU mL^{-1} samples were dispensed into a single yellow tube (mode A) or into *n* tubes (mode B). The yellow tubes were incubated for a specified period. Sampling was undertaken *n* times from the mode A culture, and once from each of the *n* cultures in mode B. Following incubation for about 16–24 h in the presence of T1 phage, the resistant colonies on each plate were counted.

have developed from resistant cells in the inoculation solution. Regardless of the probability distribution followed, this represents a statistically deterministic view, and does not consider mutant modes (spontaneous or induced). Consequently, fluctuation analysis of resistant colonies under different culture and sampling conditions can determine whether or not the fluctuations data from random experimental errors, but does not exclude the possibility of mutations arising after phage exposure.

Microbiology and molecular biology studies reveal that, apart from severely lethal effects such as high temperature and extraordinary physical damage, flora death induced by selective damage is a dynamic process, determined by the combined action of selective factor dose and treatment time. Under selective damage, the differences in hereditary and physiological characteristics between resistant and sensitive cells are too complex to be generalized as "all or nothing" [34–40]. From research on the interactions between bacteria and phages/drugs, it is known that the probability of bacteria being attacked by phages (or drugs) depends on phage density (or drug concentration) and action time. Phage multiplication is influenced by adsorption rate, lysogenic rate and latent period, as well as nonliving factors such as temperature and bacterial spatial distribution pattern [28]. Thus, when *E. coli* B cells are incubated on LB plates and exposed to T1, the possibility of induced mutation cannot be excluded. Resistant cells pre-existing in the incubation flora will grow immediately after they have been affixed to the agar surface, where they form large visible colonies after culturing for 12–18 h. Resistant cells that emerge after exposure to selection pressure produce colonies much later in the culturing period (about 18–24 h), which become visible colonies after 30 h of culturing, and thereafter increase in size. Though Luria and Delbrück, and Newcombe, noted that colonies from initially resistant cells differ in size and transparency from those appearing at later culture times, neither team offered an explanation of these differences [2,3]. In our previous studies of the relationship between antibacterial drugs and the occurrence and propagation of bacterial resistant mutants, we observed that resistant colonies varied in size and that small colonies invariably appeared at later culture stages [34–43]. Assuming that one bacterial cell multiplies into 1×10^7 cells after 23–24 divisions at 37°C, a single mutation should occur within 8–12 h, which can become a visible colony within the next 8–12 h [25,26]. During this time, resistant cells pre-existing in the incubation solution will have already formed large colonies. Thus, numerous small colonies appearing at later culture stages provide putative, but not conclusive, evidence of mutagenic effects. The multiplication and jackpot of spontaneous random mutations during pre-incubation can indeed cause fluctuations in resistant colony number of the type observed by Luria-Delbrück, but the conclusion that spontaneous mutation alone accounts for colony development post-exposure to T1 does not follow.

Based on the above argument, the development mechanism of colonies appearing on plates laced with antibacterial drugs can be statistically analyzed. Statistics is a vital tool for interpreting biological results. However, proper statistical dissection of the Luria-Delbrück fluctuation experiment will enable more informed use of statistical analysis methods in complex biological problems.

1.3 Limitation of Luria-Delbrück statistic analysis inference

Mean and variance are important descriptors of population statistics; the mean is the value around which the population is concentrated, while the variance represents the dispersion around the mean. For normally distributed data, the mean (given by $\overline{x} = \sum x_i / n,$, where x_i is an observed value and n is the sample size) represents the central point of observed values, while the variance (designated $S^2 = \sum (x_i - \overline{x})^2 / (n-1)$) specifies the extent to which individual observations are offset from the mean. Variance is the most widely used indicator of data dispersion. The goodness of fit (Pearson chi-squared test, or χ^2 as it is generally understood) is used primarily to test whether a given sample complies with hypothesis. The ratio of variance to mean (σ^2/λ), also known as the dispersion index or deviation index, determines whether small sample data ($n \cdot \overline{x} < 30$) obey the Poisson distribution. This concept is based on χ^2 and is used for nonparametric testing of whether discrete random variables expected to satisfy $S^2 = \overline{x}$ [44–50]. Usually the distribution is assumed Poisson if $\chi^2=\sigma^2/\lambda \leqslant 1.0$, and non-Poisson otherwise. The $S^2 = \overline{x}$ ratio used by Luria and Delbrück was obtained from numerous sample data. Somewhat misleadingly, the authors regard this ratio as an unbiased estimate of the population dispersion index σ^2/λ, and infer the Poisson distribution if it equals unity.

In addition, when conducting their analysis, Luria and Delbrück use the magnitude Y_i, rather than frequency y_i, of observed value. In terms of y_i, which defines the frequency with which Y_i falls into a particular interval [44–50], chi-squared is calculated as $\chi^2 = \sum (y_i - np_i)^2 / np_i$, where p_i is the expectation value of y_i, representing the theoretic frequency of a quantity of interest. In terms of Y_i, $\chi^2 = \sum (Y_i - \hat{Y}_i)^2 / \hat{Y}_i$, where $\hat{Y}_i$ is the expectation value of Y_i, representing the theoretic magnitude of an experimental quantity. The use of Y_i in the chi-squared test precludes the Luria-Delbrück approach from distinguishing probability distribution types.

2 Re-count and re-analysis of Luria and Delbrück's data

2.1 Examination of abnormal values

An abnormal value is an extreme value of univariate data, which clearly deviates from the other data in the sample. Abnormal values severely distort the results of statistical tests [51–54]. Although extreme values were predicted and noted in Luria and Delbrück's fluctuation experiment, these were not properly dispensed with during their statistical analysis. Such an omission would inevitably introduce errors into the final results.

When the population variance σ^2 is unknown, numerous methods are available by which to detect abnormal values. However, the calculations are complicated, and most of the methods are not suited to sample sizes below 10 (n<10). In this paper, abnormal values are detected via use of the Grubbs Principle [51].

The Grubbs Principle specifies an upper and lower limit, designated the Grubbs value of the upper side $g_n = x_n - \overline{x} / S$ and the Grubbs value of the lower side $g_1 = \overline{x} - x_1 / S$, respectively. If $g_n \geqslant G(n, \alpha=0.01)$ denotes a high-end abnormal value, then $g_1 \leqslant G(n, \alpha=0.01)$ is the corresponding low-end abnormal value [50–52]. Accordingly, among the bacterial colonies (comprising 310 observational data), 13 samples exhibited abnormal values in the L-D fluctuation experiment. All of the aberrant samples existed at the high-end abnormal value (Table 2). These high-end

Table 2 Detection of abnormal values in the Luria-Delbrück data using the Grubbs principle[a)]

Group A

Sample No.	A-1	A-2	A-3
Mean ($\overline{x}$)	16.7	51.4	3.3
Variance (S^2)	18.23	38.71	3.34
Standard deviation (SD)	4.27	6.22	1.83
High-end abnormal values $G(n, \alpha=0.05)$ [a]	26.99	66.39	7.71
Maximum	26	66	7
Low-end abnormal values	No	No	No

Group B

Sample No.	B-1	B-2	B-3	B-4	B-5
Mean ($\overline{x}$)	26.78	24	62.4	26.2	44.2
Variance (S^2)	1400.94	114.57	3958.71	2410.18	1325.7
Standard deviation (SD)	37.43	10.70	62.92	49.09	36.41
High-end abnormal values $G(n, \alpha=0.01)$ [a]	113.62	47.75	214.04	144.51	98.45
Maximum	125	41	183	165	107
Low-end abnormal values	No	No	No	No	No

Group C

Sample No.	C-1	C-2	C-3
Mean ($\overline{x}$)	11.35	30.58	3.79
Variance (S^2)	752.13	7542.27	43.84
Standard deviation (SD)	27.43	86.85	6.62
High-end abnormal values $G(n, \alpha=0.01)$ [a]	93.64	228.39	23.45[b]
Maximum	107	303	19
Low-end abnormal values	No	No	No

Group D

Sample No.	D-1	D-2
Mean ($\overline{x}$)	10.1	28.6
Variance (S^2)	6256	6432
Standard deviation (SD)	79.1	80.2
High-end abnormal values $G(n, \alpha=0.01)$ [a]	250	300
Maximum	500–1000(1)	201–500(4)
Low-end abnormal values	No	No

a) a, Critical maximum $G(n, \alpha=0.01)$ was calculated by Grubbs formula, $g_n = (X_i - \overline{x}) / SD$; $G(n, \alpha=0.05)$ or $G(n, \alpha=0.01)$ can be found in mathematical tables in standard statistical books. b, In sample C3, $G(n, \alpha=0.05)$.

abnormal values would significantly impact upon $\bar{x}$ and S^2, and could account for, at least partly, the uncertainty in Luria and Delbrück's data. However, since the Grubbs Principle also has its limitations, abnormal values were analyzed from a second perspective, the Stem-and-Leaf Plot method.

2.2 Sample homogeneity in the Luria-Delbrück experiment — variance analysis

The four groups, A, B, C and D, collectively contain 13 samples, and the parameters $\bar{x}$, S and S^2 differ between the samples (Table 2). Variance analysis (using one-way analysis of variance) reveals that differences between the four groups are significant (Table 3). This significant difference remains following the removal of the high-end abnormal values (table omitted). Given that the samples were taken from the same population, why are the sample variances so different? Do the differences arise from random sampling anomalies or systemic errors? Residual approaches and normal QQ plot are not appropriate for detecting the presence of systematic errors, because the data are derived from a single sampling.

Table 3 Variance analysis of the Luria-Delbrück data (Bartlett's test) [54][a)]

Sample	Bartlett's statistic	*P*-value	Does variance exist significant differences (P<0.05)?
A-1	9.016	0.0110	Yes
B-1	26.58	<0.0001	Yes
C-1	65.50	<0.0001	Yes
D-1	16.88	0.0007	Yes

a) A1, B1, C1 and D1 are representative examples, and the other samples behave similarly (omitted).

2.3 Examination of probability distribution type to observation data

2.3.1 Primary depiction of the probability distribution of the Luria-Delbrück data by Stem-and-Leaf Plot method

Data distribution may be readily visualized in a histogram. The data are binned into a number of equally-spaced intervals whose magnitudes give a coarse-grained picture of the overall probability density. The group interval largely affects the histogram shape. If the group interval is too small and the frequency of each group is too low, the frequency of nearby regions might appear large due to random effects. If the group interval is too large, the histogram is too coarse to reflect the true probability density. Thus, when the number of degrees of freedom is less than 10, the data cannot be grouped properly in a histogram. Stem-and-Leaf Plot is a schematic representation of ungrouped raw data, with the appearance of a side-on histogram. If the frequency's peak value lies centrally, with other frequencies distributed symmetrically on either side, the data follow a normal distribution. Stem-and-Leaf Plot can not only show the distribution, but can retain the individual information contained in raw data. Stem-and-Leaf plot is an effective tool for exploratory data analysis. As shown in Table 4, of nine sam-

Table 4 Analysis of the Luria-Delbrück data by Stem-and-Leaf plot [55]

Sample A-1

Stem (tens digit)	Stem (single digit)	Frequency
1	3, 3, 4, 4, 5, 5, 5	7
2	0, 1, 6	3

Sample A-2

Stem (tens digit)	Stem (single digit)	Frequency
4	4, 6, 7, 8, 9	5
5	1, 2, 6	4
6	1	1

Sample A-3

Stem (tens digit)	Stem (single digit)	Frequency
1	1	1
2	4, 2, 2, 2, 2	4
4	4, 4, 4	3
5	1	1
7	1	1

Sample B-1

Stem (tens digit)	Stem (single digit)	Frequency
0	3	1
1	0, 0, 4, 7, 7, 8	6
2	7	1
12	5	1

Sample B-2

Stem (tens digit)	Stem (single digit)	Frequency
0	7	1
1	7, 7	2
2	0, 9	2
3	0, 1	2
4	1	1
18	3	1

Sample B-3

Stem (tens digit)	Stem (single digit)	Frequency
1	0.2	2
2	3	1
3	0	1
4	0, 5	2
5	1, 7	2
17	3	1

Sample B-4

Stem (tens digit)	Stem (single digit)	Frequency
0	5, 6, 6, 8	4
1	0, 0, 3, 5	4
2	4	1
16	5	1

Sample B-5

Stem (tens digit)	Stem (single digit)	Frequency
1	3	1
2	8	1
3	5, 8	2
10	7	1

Sample C-1

Stem (tens digit)	Stem (single digit)	Frequency
0	0(10), 1, 1, 2, 5, 6	15
3	5	1
6	4	1
10	7	1

Sample C-2

Stem (tens digit)	Stem (single digit)	Frequency
0	0(5), 1, 1, 3, 4, 7	10
4	8	1
30	3	1

Sample C-3

Stem (tens digit)	Stem (single digit)	Frequency
0	0(12), 1, 1, 8	15
1	1, 5, 7, 9	4

Sample D-1

Stem (tens digit)	Stem (single digit)	Frequency
0	0(57), 1(20), 2(5), 3(3),4(3), 5, 6–10(7)	95
2	21–50(2)	2
10	1–20(1)	2
50	50–100(1)	1

Sample D-2

Stem (tens digit)	Stem (single digit)	Frequency
0	0(29), 1(17), 2(4), 3(3), 4(3), 5(2), 6–10(5)	63
1	11–20(6)	6
2	21–50(4)	4
5	51–100(5)	5
10	101–200(2)	2
20	201–500(4)	4

ples belonging to four groups (A, B, C and D), the peak frequencies accumulate at the top of the plot (on the left side of the histogram), except for the four Group B samples (where degree of freedom <10 and the data show a discrete distribution). Clearly, none of the samples are normally distributed. At least one high-end abnormal value exists in each sample.

2.3.2 Luria-Delbrück fluctuation data do not follow the Poisson distribution

Numerous methods are available by which to determine the probability distribution of discrete serial data from the same population [44–53]. Data from different populations, on the other hand, are difficult to analyze in this way. Taking the five Luria-Delbrück datasets as an example, we note that some groups (such as Group A, B and C) contain few samples, while others (e.g., D-1, D-2) nearly half of the value are 0, and many groups have high-end abnormal values. When conducting a compatibility test, we assign group intervals empirically, many uncertainties that cannot be taken into account, such as the existence of high-end abnormal values and random sampling errors. When discrete testing data from different populations does not fit the comparison condition, estimating their probability distribution by χ^2 method is a complicated task.

χ^2 can be determined in a number of ways depending on the distribution types, however, the calculations are relatively intricate. The formula is given by $\chi^2 = \sum(O_i - T_i)^2 / T_i$ [47], where O_i and T_i denote the observed and theoretical frequency, respectively, of the *i*th datum. The theoretical frequency is $T_i = P_i \cdot \sum O_i$, where P_i is the expected value of the theoretical distribution probability. Like the Poisson distribution, the expected value $(P_{(k)})$ of the theoretical probability distribution is $P_{(k)} = \frac{\lambda^k \cdot e^{-\lambda}}{k!}$. In general, χ^2 increases as the interval of the data grouping decreases. When the number of samplings is less than 30, neighboring groups must be combined so that the expected frequency is not less than 5. The assignment of χ^2 depends on the number of degrees of freedom of the grouping, and accurate judgment is difficult.

The four Luria-Delbrück datasets show that the presence of the previously-mentioned uncertainties would incur high error rate. Applying the above method to the data (Groups A, B, C and D) in an attempt to discern the probability distribution type, large differences in χ^2 are obtained. Changing the group interval alters or even reverses the results. Because the abnormal value cannot be arbitrarily removed, being considered as a systematic error, we attempt to solve the problem by constructing a histogram of the relative probability distribution. The frequency distribution graph is then derived, from which we hope to glean an intuitive understanding of the data's distribution trend. Next, plot ln(p_i, k!) as a function of k!. If the resulting points form a straight line, an upward curve or a downward curve, the distribution is judged as Poisson, negative binomial (aggregated distribution) or binomial, respectively [53,54]. This idea is rooted in the theory of probability: given a random variable $\zeta \sim P(\lambda)$, then $P(\zeta = X) = \frac{e^{-\lambda}\lambda^X}{X!}$, where k=0, 1, 2, ..., λ>0.

Suppose that data come from a Poisson population whose argument is λ, then in n trials the theoretical frequency of ζ equal X is $np_i = n \cdot \frac{e^{-\lambda}\lambda^X}{X!}$. Taking the logarithm, $\ln(p_i x!) = -\lambda + x \ln \lambda$. Thus, the point [$X$, ln($p_i x$!)] lies on a straight line with slope lnλ and intercept $-\lambda$. Because measured frequency in fact fluctuates around the expected frequency, the points (X, ln($p_i x$!)) will scatter above and below the theoretical line. The resulting Poisson Graph can assist our judgment of whether data follow the Poisson distribution. A linear Poisson Graph is indicative of a Poisson population. The population mean λ can then be estimated by $\lambda = \overline{x} = \frac{1}{n}\sum_i n_i x_i$. Alternatively, some of the isolated points which lie far from the theoretical line can be removed,

and a new straight line fitted. The slope b now provides a reasonable estimate of λ through the relationship $\hat{\lambda} = e^b$, the exponential form of the slope $\ln \hat{\lambda} = b$. If the Poisson Graph is non-linear, the data are not derived from a Poisson population.

Both methods gave consistent results when applied to the Luria-Delbrück data (omitted). Figures 2 and 3 are the results of fitting five datasets selected from Groups A, B, C, D-1 and D-2 of the Luria-Delbrück experiment. From the relative frequency histogram of Figure 2, we observe that the χ^2 s aggregate towards the left. The general distribution is left-tilting and is the classic probability distribution type of χ^2.

$\ln(p_i x!)$ as a function of $x!$ is plotted in Figure 3. In this figure, the scattered points curve upwards, indicating that all σ^2/s ratios exceed 1.0. The curve is well-fitted to a quadratic function, conforming that the data fit the negative binomial distribution (aggregated distribution) or binomial distribution [53,54], rather than the Poisson distribution.

The negative binomial distribution has different distribution of the probability from the Poisson distribution. Index k defines the aggregation degree, the smaller than 1 of k, the greater the degree of aggregation and the wider the variance; while in Poisson distribution, the variance retains the same as the average, and k equals 1.

2.3.3 The Luria-Delbrück fluctuation data are consistent with aggregated distribution

Aggregated distribution is a form of negative binomial distribution commonly found in bionomics. The degree of gathering is a crucial component of spatial analysis, and is encountered in many forms in probability models describing the random distribution of biological populations [53–55]. Two examples will suffice to illustrate the various forms of the distribution.

(i) Lloyd's average gathering degree model. This is defined by $m' = (m + S^2)/(m-1)$, where m' is the average gathering degree index, m is the average value and S^2 is the variance. If the gathering index $m'/m > 1.0$, the distribution is said to be aggregated.

(ii) Moment method. This form is focused on the gathering degree k, defined as

$k = \bar{x}^2/(S^2 - \bar{x})$, $\bar{x} = 1/\ x\sum f_i \cdot i$, $S^2 = \sum f_i \cdot i(i-\bar{x})^2/(N-1)$. Here, i is the magnitude of an observed value, f_i is the frequency of i, and N is the sample cases. The aggregation test reveals that in five of the Luria-Delbrück groups, Groups A, B, C, D-1 and D-2, the data are aggregated (Tables 5 and 6). The datasets show different values of the gathering degree k. By this token, it appears that there are biases when Luria and Delbrück judged the probability

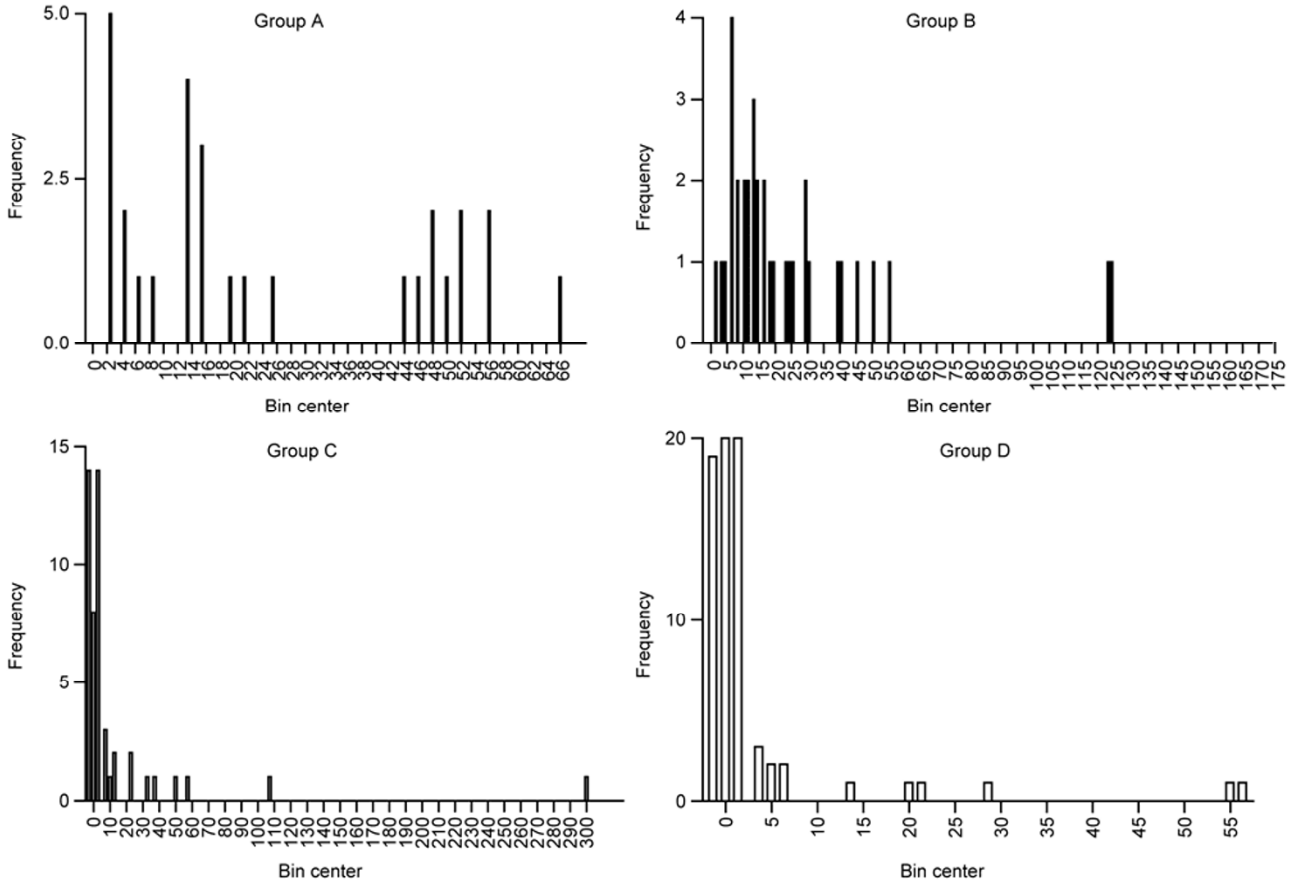

Figure 2 Frequency distribution histogram of the Luria-Delbrück data.

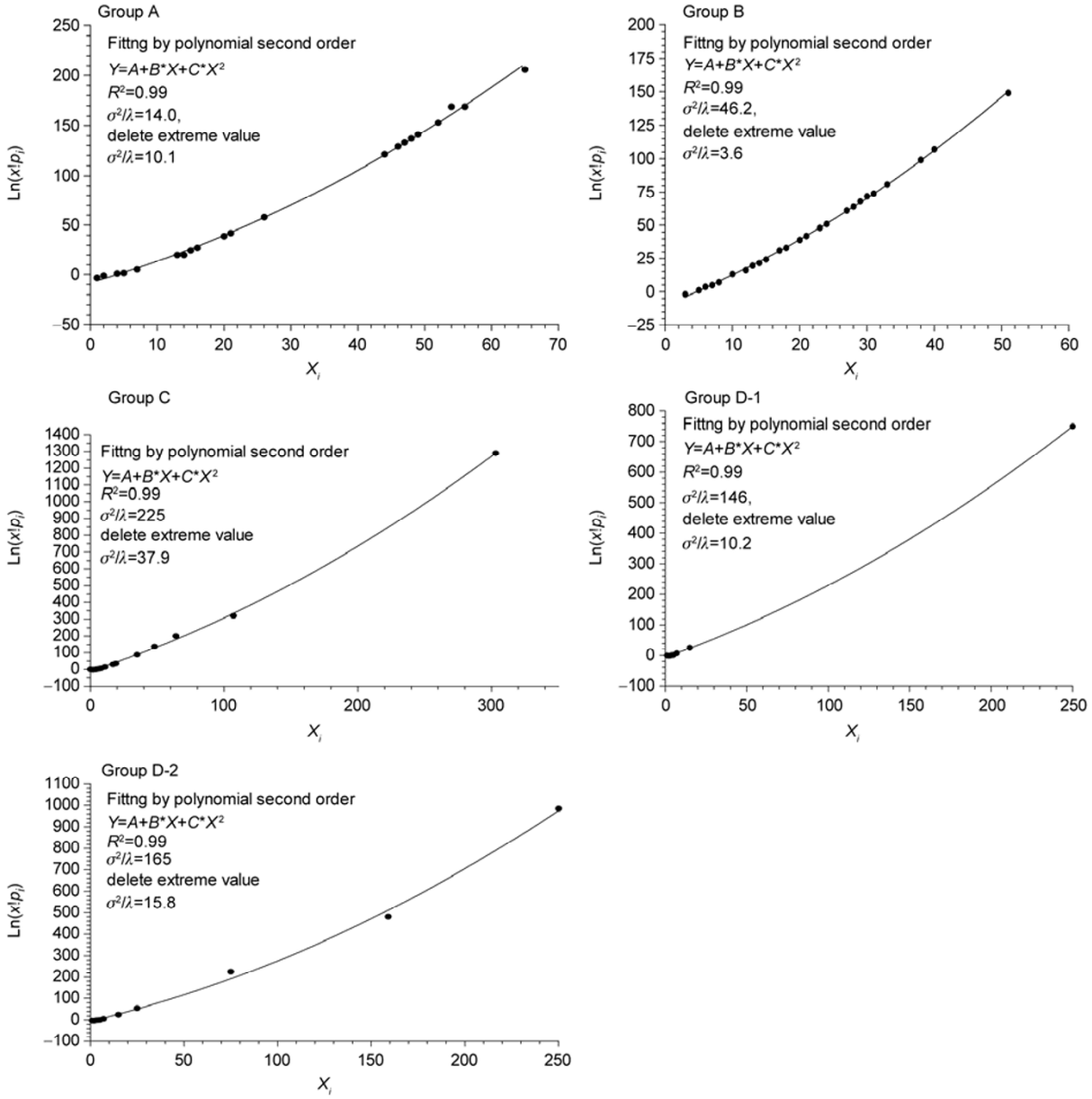

Figure 3 $\ln(x!p_i)$ vs. X_i plots for five different Luria-Delbrück datasets, used to determine the probability distribution type of the discrete data.

Table 5 The aggregation degree k for five Luria-Delbrück groups, estimated by moment method

Groups	A	B	C	D1	D2
$\overline{x}$	2.5	1.7	2.6	9.9	7.5
S^2	37.4	27.0	47.0	30.6	69.6
$S^2/\overline{x}$	14.9	15.6	7.7	3.1	9.3
k	0.18	0.11	0.16	0.36	0.91

distribution type of the five datasets by estimating the extent of deviation from the Poisson distribution.

3 Possible cause of aggregated distribution

Microbiological and genetic analyses have shown that spon-

Table 6 Estimation of aggregated distribution in the Luria-Delbrück groups (using Lloyd's average gathering degree model)

	m	S^2	m'	m'/m and mean	
A1	16.5	5.9	16.9	1.02	
A2	50.6	15.0	50.9	1.01	1.04
A3	3.37	0.71	3.67	1.1	
B1	26.8	24.5	36.9	1.35	
B2	22.5	107	27.5	1.22	
B3	52.1	580	63.1	1.21	1.22
B4	26.2	200	34.1	1.30	
B5	44.2	468	45.1	1.02	
C1	11.1	618	72.1	6.5	
C2	31	4529	181.6	5.86	5.65
C3	4.3	51	19.7	4.59	
D1	9.7	813	102.8	10.6	
D2	28.6	810	60.1	2.1	

taneous and induced bacterial mutations are rare events. Typically, when a sensitive bacterium divides into 1×10^7 clones, a single random mutation may occur. In culture solutions, induced mutations are feasible among the millions of sensitive bacterial cells. The test data of Luria and Delbrück indicate an average mutation rate of 2.45×10^{-8} per bacterium per cell division. That is, when the sensitive bacteria multiply to 1×10^8–5×10^9 mL^{-1}, colonies will form from spontaneously mutated cells. However, around every mutated cell, billions of sensitive cells reside at high density. Steric and competition effects are therefore inevitable. Such spatial restrictions prevent the mutated cells from spreading randomly and induce aggregated distribution. Thus, homogeneous sampling might intensify the extent to which resistant bacterial colonies vary from plate to plate. When 0.5 or 0.02 mL of culture containing mutated cells is spread onto the LB agar surface, the resultant colony derives from either an individual resistant cell or a cluster of resistant cells. Therefore, the number of colonies on the plate depends to some extent on the homogeneity of the spreading. Resistant cells, which have different gathering degree and which are surrounded by sensitive cells, might be particularly prone to this effect.

However, Newcombe's re-spread experiment indicates clearly (Table 7) that counts of phage-resistant bacterial colonies increase significantly following re-spreading of resistant colonies [3]. In this test, multiple liquid bacterial inoculums are spotted (fixed-spot inoculation) on the surface of an LB agar plate using a special inoculator. The spots are evenly spaced to ensure that they spread without merging for up to 6 h. Phage is added and the plates are cultured for a further 24 h, followed by counting the number of resultant colonies (Table 7). In the unspreading group, the number of resistant colonies increased exponentially by 30 times (from 1.33 to 40) within 4–6 h. In the spreading group, colony count followed a type of Boltzmann-Sigmoidal pattern, increasing 1624 times (from 1.33 to 2160) within the same time period. Newcombe considered this as evidence that the emergence of T1 phage resistant mutants in Luria and Delbrück's data were independent of T1 phage presence. What Newcombe did not explain was the vast difference in the number of resistant colonies between the two groups. Of researchers who have since quoted Newcombe's result, none have considered this phenomenon. We regard the result as affirmation that T1 phage can induce mutation, and is a manifestation of mutant phenotypic expression. We assert this idea because large numbers of sensitive cells present in inoculation spots can produce more resistant cells than spontaneously mutated cells. Although these resistant cells multiply as the culturing time increases, they are fixed on the agar surface and limited to the regions of fixed-point inoculation. During re-spreading, the abundant resistant cells accumulated within the colonies of the surface can freely separate and form new colonies, with corresponding large increases in resistant colony counts. If the resistant colonies in the re-spreading test arise solely from the resistant cells within inoculation spots, an exponential increase of 40 times is expected, rather than the observed increase of 1624 times.

Luria and Delbrück expected that, in all of their groups, 10 to 100-fold differences in pre-cultured inoculations could account for the large variance-mean ratio observed in the colonies ($S^2/\bar{x}$). This interpretation could deduce that mutant strains in the Luria Delbrück experiment were not correlated with phage presence. From the above-mentioned information, we proceed with a detailed analysis. In the

Table 7 Analysis of Newcombe's re-spreading test results [3][a)]

Incubation time (h)	3 h		4 h		5 h		6 h	
Bact. plated	5.1×10^4		5.1×10^4		5.1×10^4		5.1×10^4	
End No. bact.	1.7×10^6		2.3×10^7		2.6×10^8		2.8×10^9	
Factor increased	33		480		5100		54900	
Resistant colonies	Unsp	Sp	Unsp	Sp	Unsp	Sp	Unsp	Sp
Replatica test 1	0	0	0	0	5	194	46	2254
2	0	0	3	0	3	14	25	1434
3	0	1	0	6	4	16	45	3294
4	0	0	2	0	8	13	49	3719
5	0	0	1	0	2	4	26	1538
6	0	1	2	2	6	112	49	399
$\sum x_i$	0	2	8	8	28	353	240	12638
$\bar{x}$	0	0.33	1.33	1.33	4.67	58.83	40	2106.3
SD	0	0.52	1.21	2.42	2.16	77.52	11.35	1242.83
S^2	0	0.27	1.47	5.87	4.67	6009.8	128.8	1544615
$S^2/\bar{x}$	–	0.8	1.1	4.4	1	102.15	3.22	733.32

a) Unsp, un-spread; Sp, spread.

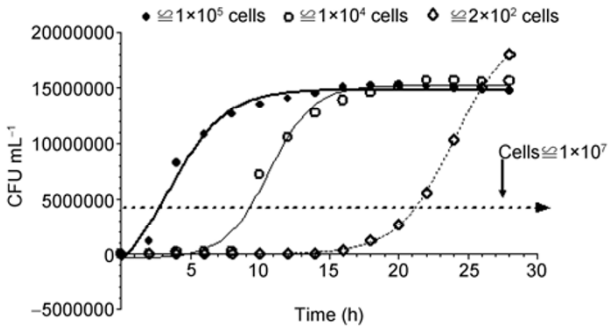

Figure 4 Kinetics growth curve of *E. coli* CVCC249 (37°C). The cell numbers at the top of the figure are the inoculum sizes. The curve is an analog of Boltzmann-sigmoid, R^2=0.98.

following, we use bacterial colony growth kinetics to analyze how a 10 to 100-fold difference in initial inoculums can influence the emergence of spontaneous mutant strains. The growth of the strain *E. coli* CVCC249 is displayed in Figure 4. Starting from 1×10^5, 1×10^4, or 2×10^2 CFU mL^{-1}, the strain was inoculated into a 7 L fermentation tank containing 5 L LB liquid medium at 37°C. The strain was sampled at one-hour intervals during the first 12 h, and at two-hour intervals thereafter. At least three samples were taken per time point, and the CV (coefficient of variation) was found to be less than 5% [35]. The kinetics curve of batch culture approximates closely to the Boltzmann-Sigmoid curve (R^2=0.98). Applying the Boltzmann-Sigmoidal equation to an inoculation of 1×10^5 cell mL^{-1}, the cells should number 1×10^7 after 3.5 h, sufficiently high for a probable spontaneous mutation. When the inoculation is 1×10^4 or 2×10^2 cell mL^{-1}, however, a spontaneous mutation is unlikely to appear until 15 and 24 h have lapsed, respectively. At 24 and 32 h, the 1×10^5 CFU mL^{-1} inoculum contained more mutated cells than the other two inoculums, probably due to differences in sampling volume. Luria and Delbrück similarly observed that large colonies would appear within 12 h, but small colonies did not emerge until 24–28 h [2]. From Figure 4, we consider that large colonies arise from spontaneous mutation during pre-culturing, while the late-appearing small colonies are responses to phage presence on the plate. Thus, the possibility of induction cannot be excluded.

4 Discussion

4.1 The distribution of mutated cell numbers in Luria and Delbrück's fluctuation test is not Poisson

The mutant cells in the liquid culture environment of Luria and Delbrück's fluctuation test arise in two ways. Cells can be newly mutated, or are the progeny of a previously mutated cell. In cells arising from the first source, the number of mutant cells reflects the number of mutations, and should obey the Poisson distribution. This view is widely accepted by biologists and mathematicians [44,45,60]. In the classic test of adaptive mutations presented by Cairns and other researchers in 1988 (where $lacZ^-\rightarrow lacZ^+$ reversion mutants grew using lactose as the sole carbon source), a single colony appeared per mutation event. Therefore, Cairns's test appeared to confirm that colonies result from mutations, and are therefore Poisson variables [6]. Different from the Luria-Delbrück distribution, Cairns's results complied with the Poisson distribution [6]. Such conclusions ignore the second mechanism by which mutant cells emerge in liquid solution. In fact, the number of mutant cells in Luria and Delbrück's fluctuation test depends not only on recent mutations, but also on time. The longer the interval between the appearance of a mutation and the time of spreading, the more generations the mutant cell can produce. One definition of a Poisson event is that "Poisson event is accidental event, and has nothing to do with the existing quantity and time" [46]. The Poisson distribution describes the probability distribution of discrete and random events. "Whether the random event will happen at a particular time or not is not only independent of time, but also independent of the occurred frequency" [61]. We have shown that the number of mutant cells in liquid culture does not fit the Poisson distribution, and whether the number of mutant cells in liquid culture fit the Poisson distribution cannot be used as the criterion to distinguish spontaneous and induced mutations. The statistician Chen XiRu pointed in his book *Mathematics of Probability* that "Statistical regulations may not contain causal relations. The search of causal relations is the duty of every subject" [62]. Our conclusions support this sentiment.

4.2 In the fluctuation test of Luria and Delbrück, induced mutation may conceivably occur after exposure to a fatal selective factor (phage T1)

Spontaneous mutation is a rare event under no-choice conditions. In the fluctuation experiment, culturing prior to spreading will increase the numbers of spontaneously mutated cells to varying extent. When such cells are inoculated under excessive selective pressure (for example, in the presence of T1 phage), the restrained growth of sensitive cells and their death are temporal processes. Since the flora is not uniformly sensitive to the selective condition, the probability of an induced mutation is significant. The colonies that ultimately appear on the culturing plate should result from both spontaneous mutation during pre-culturing and induced mutation following exposure to phage. The aggregated distribution of mutant cells and the existence of large and small colonies reflect this mechanism. Admittedly, the probability of induced mutation by phage T1 presence does not conflict with the opinion of Luria and Delbrück that spontaneous mutation could appear prior to phage exposure.

4.3 Understanding the Luria-Delbrück fluctuation test from a mathematical statistics perspective

Luria and Delbrück noted that large and small flora appeared at different times in the culturing period, and reported that the morphological and growth features are similar between the two colony types. Liu [36,41] reported *E. coli* resistant colonies on plates containing antibiotics (Enrofloxacin) had different-diameters, and the cells within the colonies with similar genotype. Jin [63] reported that reversion mutants of *E. coli leu*B$^-$ exhibit three distinct genotypes, whether they were resulted from mutations under non-selective medium or selective medium. In addition, the three different revertants appeared in approximately equal proportion. Therefore, the probability distribution of mutant bacteria colonies is not the only means of assessing mutation mechanisms.

The sampling error and the uniformity of spreading may exert considerable influence on data accuracy, yet these factors are often ignored. In calculating the rate of bacterial mutations, different methods yielded different results. For example, repeated sampling was applied to Luria and Delbrück's Group A dataset, in which the sample was derived from approximately half the sample volume. Using this method, the number of mutated cells (excluding the influence of distribution) was taken as the average of 10 samplings. This average should be close to the true value. For Groups B, C and D, samples were randomly extracted from 1/10 to 1/20 of the sample volume, and the number of mutated cells was calculated once only. Such randomness will contribute to the error in this method. Consequently, many nulls and high-end abnormal values were present in the Group C, D-1 and D-2 data. Because the sampling errors and both factors of aggregated distribution have been omitted from the analysis, a statistical analysis based on randomness and uniformity is scarcely possible. Meanwhile, the mutant cells sampled from the culturing solution tend to aggregate, so that numbers of resistant colonies are inextricably linked to the uniformity of spreading on the selective plate. A standardized operational method, which would reduce the spreading effect, is not used. Herein lies the problem of calculating mutation rate from counts of bacterial colonies on selective medium.

The high-end abnormal values encountered in Luria and Delbrück's fluctuation data may result from cell clustering during cell division and increase. Because mutated cells give rise to mutated daughter cells, the emergence of the mutant population is time-dependent. A characteristic of the Poisson distribution is that the arithmetic average value is the unbiased estimate of the expected value. The ratio between arithmetic average value and variance is also indicative of the Poisson distribution. Applying these tests to Luria and Delbrück's fluctuation data, we established that the number of mutated cells in liquid solution does not comply with the Poisson distribution, and that an unacceptable number of high-end abnormal values exist. Assuming the arithmetic average value as the expected value would incur large errors.

The mutated cell data of Luria and Delbrück's fluctuation test have been revealed as aggregated distribution. This is consistent with Liu Shi's [25] hypothesis that isolated *E.coli* cells do not immediately separate after cell division. The non-separation of divided cells may be a major cause of such aggregated distribution. Furthermore, since repeated and non-repeated sampling methods were applied to Group A and the remaining groups, respectively, the mean to variance ratios cannot be compared between the experimental groups.

4.4 Drug resistance in bacteria

A drug may destroy cells in a non-selective fashion, may target a specific gene or group of genes, or may exert a mutagenesis effect on multiple targets. Depending on the conditions, the drug may physiologically alter the bacterial cells, reduce the copying capability of the bacterial DNA (or the fidelity during copying), or may damage the ability of the DNA to correct impairments (for example, interfering with the SOS response). Thus, during exposure to drugs, the spontaneous mutation rate of bacteria increases and various mutations may appear with high frequency. It is known that mutation of phenotypes is random [57–61,63], but we cannot deduce reversely that spontaneous mutation occurs prior to drug exposure.

4.5 Summary

Throughout the past 60 years, the deductions of Luria and Delbrück have been accepted without analysis of the casual relations between the emergence and increase of resistant mutated strains and selective damaging agents. Instead, mutation rate has been estimated by applying different probability analysis methods. From a microbiological and growth kinetics perspective, when the growth and mutation occurrence of a bacterial strain cannot be tested directly, the mutation rate is difficult to estimate and characterize [9,25–29,34–39]. What previous researchers have actually obtained is the number of resistant colony forming units, which is not a strong indicator of special mutation [63]. The controversies over adaptable versus spontaneous mutation have remained for more than 20 years. Because adaptive mutation involves the very complicated regulative mechanism of SOS response, these controversies are not likely to be resolved in the near future [58–63]. Mutation appears on individual bacteria cell, but the expression of the mutant phenotype occurs during proliferation. This is a kinetic, temporally dynamic process and may be regarded statistically as the procedural function. However, the mutant bacterial colonies appearing on the plate represent a terminal value, may be regarded statistically as a state function.

Whether the resistant mutation characteristics will show or not is also linked to the presence of large proliferating mutant cells, so that focusing on the transient nature of the proliferation kinetics may be provide good method for well understanding the mechanisms by which bacteria mutate to evade a threat.

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